FORMATION AND CLEAVAGE OF 1→2 GLYCOSIDIC BONDS BY HEN'S EGG WHITE LYSOZYME

Jerry J. Pollock and Nathan Sharon

Department of Biophysics, The Weizmann Institute of Science,

Rehovoth, Israel

Received January 31, 1969

Summary: GlcNAc β(1→4)-MurNAc β(1→2)-D-Gal was prepared by lysozyme catalysed transglycosylation, and its structure established by chemical methods. Lysozyme releases D-galactose from this trisaccharide showing that the enzyme can cleave β(1→2) linkages. The formation of GlcNAc β(1→4)-MurNAc β(1→2)-D-Glc, in addition to the corresponding β(1→4)-D-Glc trisaccharide has also been demonstrated. In the case of D-xylose, corresponding trisaccharides with β(1→2)-, β(1→3)-, and β(1→4)-D-Xyl linkages were found. These findings are correlated with the three dimensional lysozyme-substrate model.

The transglycosylase activity of hen's egg white lysozyme (1) has been used to investigate the substrate specificity at subsites E and F (2, 3) in the active site of the enzyme, and as a tool for studying other aspects of the mechanism of lysozyme action (4), but the nature of the new saccharide linkages formed during the transfer process is unknown. It has generally been accepted that β(1→4) glycosidic linkages are formed since lysozyme substrates are attached through such bonds (1). However, close examination of the three dimensional model of lysozyme (5), especially of subsite E, might allow one to suggest that lysozyme may catalyse the formation, as well as cleavage, of linkages other than β(1→4), depending on both saccharide structure and steric requirements imposed by the enzyme. This communication presents evidence on the lysozyme catalysed formation and breakdown of trisaccharides containing linkages other than (1→4). Some aspects of the specificity of subsite E in relation to the three dimensional lysozyme-substrate model (5) are also considered.

Materials and Methods: Hen's egg white lysozyme (twice recrystallized, salt free, Lot LYSF 71A) was a product of Worthington. The tetrasaccharide, GlcNAc β(1→4)-MurNAc β(1→4)-GlcNAc β(1→4)-MurNAc (either labeled or unlabeled, where GlcNAc is N-acetyl-D-glucosamine and MurNAc is N-acetylmuramic acid), was prepared as previously described (1, 4). The monosaccharides used were all commercial products.

Trisaccharides of the general structure GlcNAc β(1→4)-MurNAc-X where X is D-galactose, D-glucose, D-xylose, or GlcNAc were prepared by lysozyme catalyzed transfer of GlcNAc β(1→4)-MurNAc from the tetrasaccharide (donor) to X (labeled or unlabeled acceptor). The trisaccharides were isolated and purified by a combination of anion exchange chromatography, preparative paper electrophoresis, and preparative paper chromatography (details to be published). They were analysed for homogeneity by paper chromatography in n-butanol-acetic acid-water (25:6:25, upper phase) and ethyl acetate-pyridine-water (2:1:2, upper phase) and by high voltage paper electrophoresis in acetic acid - pyridine buffer, pH 6.5 (4) and germanium dioxide-sodium hydroxide buffer, pH 10.7 (6). For all the trisaccharides isolated, X was shown by sodium borohydride reduction to be at the reducing end of the molecule. In addition, the analyses for X (7), and for GlcNAc and MurNAc (8) in the isolated compounds were in agreement with the structures proposed.

Periodate oxidations were followed spectrophotometrically according to Leyh-Bouille et al (9), on both unmodified and sodium borohydride reduced compounds. When 1-^{14}C labeled trisaccharides were used, they were first reduced with sodium borohydride, oxidized with sodium periodate, and then again reduced with sodium borohydride. After acid hydrolysis, deionization by mixed bed resin, and evaporation, aliquots were counted by liquid scintillation and spotted on papers for descending chromatography and electrophoresis at pH 10.7 to detect labeled glycerol. The triphenyltetrazolium chloride (TTC) reaction was performed according to Schiffman et al (10).

Acceptor studies were carried out with lysozyme (0.3 mg per ml) at pH 5.25, 37°, using a molar concentration ratio of acceptor saccharide, X (5×10^{-1} M), to labeled donor, GlcNAc β(1→4)- MurNAc β(1→4)-GlcNAc β(1→4)-MurNAc (1×10^{-2} M), of 50 to 1. Radioactive products formed during the course of incubation were analysed by paper electrophoresis, pH 6.5, in the usual manner (2, 4). For testing the susceptibility of the isolated trisaccharides to lysozyme digestion, they were incubated in pH 5.25 ammonium acetate-acetic acid buffer (0.1 M in acetate) using concentrations of 2.4 to 7.9×10^{-2} M trisaccharide and 1 mg per ml enzyme and the incubation mixtures analysed as above.

Results and Discussion: As shown in Table I, the trisaccharide, GlcNAc β(1→4)-MurNAc-D-Gal-1-^{14}C migrated as a single radioactive peak in all of the paper chromatographic and electrophoretic systems used. Upon periodate-$NaBH_4$ degradation, it yielded ^{14}C-labeled glycerol as the sole radioactive product, indicating that the linkage formed by lysozyme transglycosylation between MurNAc and D-Gal is 1→2. Quantitative estimation of the periodate uptake and formaldehyde released by the unmodified and by the $NaBH_4$ reduced GlcNAc β(1→4)-MurNAc-D-Gal gave results in complete agreement with this conclusion. Furthermore, this trisaccharide (0.8 μmole per 0.1 ml) did not give a color with the TTC reagent. The (1→2)-linked disaccharide sophorose also remained colorless at the same concentration, whereas laminaribiose, cellobiose, and gentiobiose (all at 0.15 μmole per 0.1 ml) gave dark red formazan precipitates.

TABLE I

Properties of trisaccharides formed by lysozyme catalysed transglycosylation

Trisaccharide	Percent of Radioactivity[a] per Peak				Glycerol-^{14}C[b]	Digestion Experiment[e]	
	Electrophoresis pH 6.5	B:A:W 25:6:25	E:P:W 2:1:2	Electrophoresis pH 10.7		Molar Concentration	Percent of Cleavage
GlcNAc β(1→4)-MurNAc-D-Gal-1-^{14}C	100	100	100	100	+	2.4×10^{-2}	12
GlcNAc β(1→4)-MurNAc-D-Glc-1-^{14}C	100	100	100	88; 12	+	5.3×10^{-2}	2.1
GlcNAc β(1→4)-MurNAc-D-Xyl-1-^{14}C "A"	100	55	55	55	-	7.9×10^{-2}	2.1
"B"		45	45	24; 21	+	7.9×10^{-2}	4
GlcNAc β(1→4)-MurNAc-GlcNAc-1-^{14}C	100	100	100	c	d	3.7×10^{-2}	1

a - A value of 100% of radioactivity indicates that the method of analysis detects only a single peak. With some trisaccharides more than one peak is formed and the radioactivity of the peaks totals 100%.

b - For (1→2) linkages, glycerol is formed after periodate-$NaBH_4$ degradation (see text). It is detected by paper chromatography and by paper electrophoresis at pH 10.7.

c - Not tested.

d - Glycerol-^{14}C cannot theoretically be formed because of the N-acetamido group at carbon-2 of GlcNAc.

e - Trisaccharides were incubated at 37° with lysozyme (1 mg per ml) in pH 5.25 acetate buffer for 98 hours except in the case of GlcNAc β(1→4)-MurNAc-D-Gal, for which the period of incubation was only 72 hours. Percent of cleavage by lysozyme was determined from the amount of radioactive monosaccharide released following pH 6.5 electrophoresis of the reaction mixture.

B:A:W butanol-acetic acid-water; E:P:W ethylacetate-pyridine-water.

GlcNAc β(1→ 4)-MurNAc-D-Glc- 1-^{14}C, which migrated as a single peak in the paper chromatographic solvent systems, was found by paper electrophoresis, pH 10.7, to be a mixture of two components, a major one (88% of radioactivity) and a minor one (12% of radioactivity). Periodate-$NaBH_4$ degradation of this trisaccharide resulted in a small amount of ^{14}C-glycerol (about 10% of its radioactivity), suggesting the presence of a (1→ 2) linkage, probably in the minor component observed on paper electrophoresis at pH 10.7. The major product has a(1→ 4)linkage since Smith degradation (11) of GlcNAc β(1→ 4)-MurNAc-D-Glc-U-^{14}C, followed by acid hydrolysis, gave ^{14}C-labeled erythritol. The presence of both (1→ 2)- and (1→ 4)-linked glucose in GlcNAc β(1→ 4)-MurNAc-D-Glc is supported by the finding that the trisaccharide gave a red formazan precipitate with the TTC reagent, and by the results of gas liquid chromatographic studies of the glucose methyl ethers obtained from the methylated trisaccharide.

GlcNAc β(1→ 4)-MurNAc-D-Xyl-1-^{14}C migrated as two distinct components in the paper chromatographic solvents used. These two components were separated and purified by preparative paper chromatography in butanol-acetic acid-water, 25:6:25. One, designated xylose trisaccharide "A" (55% of radioactivity), was homogeneous upon electrophoresis at pH 10.7, whereas the other, xylose trisaccharide "B" (45% of radioactivity), could be further separated by electrophoresis at pH 10.7 into two peaks. No ^{14}C-glycerol was detected upon periodate-$NaBH_4$ degradation of xylose-1-^{14}C trisaccharide "A". Periodate oxidation studies as well as gas liquid chromatographic analysis of the xylose methyl ethers obtained from this trisaccharide indicated that the linkage between MurNAc and D-Xyl is (1→ 4). Upon periodate-$NaBH_4$ degradation of xylose-1-^{14}C trisaccharide "B", less than half of the original radioactivity was recovered as ^{14}C-glycerol. Although the latter result indicates a 1→ 2 linkage between MurNAc and D-Xyl, trisaccharide "B" gave a red color in the TTC test suggesting the presence of an additional linkage. That xylose trisaccharide "B" is a mixture of GlcNAc β(1→ 4)-MurNAc-(1→ 2)-D-Xyl and GlcNAc β(1→ 4)-MurNAc-(1→ 3)-D-Xyl has been substantiated by gas liquid chromatographic studies.

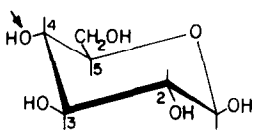
GlcNAc β(1→ 4)-MurNAc-GlcNAc migrated as a single peak in all systems used and is identical by all the criteria tested (optical rotation, periodate oxidation, chromatographic and electrophoretic properties, exo-β-N-acetylglucosaminidase susceptibility, etc.) to GlcNAc β(1→ 4)-MurNAc β(1→ 4)-GlcNAc obtained by digestion of GlcNAc β(1→ 4)-MurNAc β(1→ 4)-GlcNAc β(1→ 4)-MurNAc with lysostaphin (12).

All the trisaccharides exhibited negative optical rotations suggesting that the new linkage formed between MurNAc and X is of the β configuration. This is in accord with previous results on the retention of configuration for transfer to saccharides (13) and to methanol (3, 14).

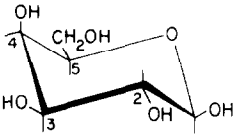
Evidence to support various linkages formed by lysozyme also came from studies of acceptor specificity. Galactose derivatives which lack the 2-OH group, such as 2-deoxy-D-galactose and N-acetyl-D-galactosamine are not acceptors (less than 1% as efficient as D-glucose), while 2-deoxy-D-glucose and D-mannose (which presumably form (1→4) linkages) are as efficient acceptors as D-glucose. The fact that 4-O-methyl-D-xylose is an acceptor further demonstrates that in the case of D-xylose, linkages other than (1→4) are possible. We have also shown that certain L-sugars such as L-xylose, L-arabinose, L-glucose, and L-fucose can serve as acceptors, while other L-sugars such as L-mannose and 6-deoxy-L-mannose (L-rhamnose) cannot.

An explanation of these results is not immediately forthcoming until one makes a close examination of the three dimensional lysozyme-substrate model (5). If D-galactose is to occupy subsite E in the same orientation as N-acetyl-D-glucosamine in the model, then its 4-OH, which is axial, would point out away from carbon 1 of the MurNAc residue at subsite D. D-galactose therefore would be unable to use its 4-OH as a nucleophile to form the same type of β(1→4) linkage as that formed during transglycosylation between MurNAc and the terminal reducing GlcNAc in GlcNAc β(1→4)-MurNAc β(1→4)-GlcNAc (12). However, our results indicate that the 2-OH of D-galactose acts as the nucleophile to form GlcNAc β(1→4)-MurNAc β(1→2)-D-Gal. One can interpret this finding on the assumption that D-galactose is positioned by the enzyme for nucleophilic attack in a different geometrical manner than GlcNAc. This new manner of binding must still meet steric requirements and allow interactions with the enzyme. It can be achieved by a 120^0 clockwise rotation by the sugar around its three fold axis of symmetry (Figure 1). As a result of this movement, the D-galactose ring will still fit snugly into subsite E, and while its 2-OH (equatorial) will be superimposed on the 4-OH of GlcNAc, its 4-OH (axial) will now lie over the ring oxygen of GlcNAc. Examination of the model of the enzyme shows that this axial group would apparently not make contact with the enzyme. Similarly, the 6-CH_2OH group of D-galactose could easily be accommodated in the cleft of the enzyme, since it would be directed into subsite F.

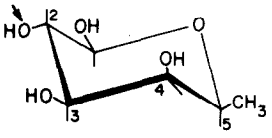
In contrast to D-galactose, D-glucose can assume the same orientation as GlcNAc, as evidenced by its predominant formation of a (1→4)-linked trisaccharide. However,



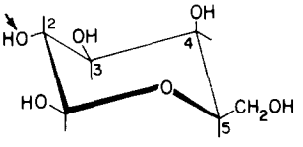
β-D-GLUCOSE



β-D-GALACTOSE-I



β-L-FUCOSE



β-D-GALACTOSE-II

FIG. I. Conformational structures of monosaccharide acceptors shown in their β forms. Carbons 2 to 5 are numbered for each of the compounds. The arrow denotes the oxygen involved in formation of the new glycosidic bond in the lysozyme catalysed transglycosylation reaction. The orientation of β-D-glucose is assumed to be like that of N-acetyl-D glucosamine in subsite E, in the three dimensional lysozyme-substrate model (5). β-L-fucose and β-D-galactose-II are drawn in a manner where their 2-OH is superimposed on the 4-OH of β-D-glucose so that they can serve as acceptors in the transfer reaction. Note that β-D-galactose-I cannot be an acceptor since its axial 4-OH points away from the position of new bond formation.

---

like D-galactose it can also rotate by 120^0 to give some GlcNAc β(1→ 4)-MurNAc β(1→ 2)-D-Glc (see Table I). In the case of D-xylose there is yet another linkage, β(1→ 3), being formed. Apparently, the lack of a 6-CH_2OH group presents the xylose molecule with an opportunity to interact with the enzyme in a completely 'unorthodox' fashion.

In the case of the L-sugars, L-glucose or L-fucose, examination of the model (see also Figure 1) shows that a (1→ 2) linkage would appear to best fulfil requirements needed for acceptor activity and we therefore assume this to be the linkage formed with these two L-sugars. When L-fucose is bound in the proposed manner to subsite E, its carbon-4 will be superimposed on carbon-2 of GlcNAc in the lysozyme-substrate model (Figure 1). However, instead of an equatorial acetamido group, L-fucose will now have an axial hydroxyl group. This is analogous to the orientation in subsite E of the axial 2-OH of D-mannose, which is assumed to be positioned like GlcNAc in the model and as mentioned is just as efficient an acceptor as D-glucose. In contrast to the above L-sugars, L-mannose or L-rhamnose do not serve as acceptors because

even if they can fit into subsite E in the manner proposed for L-fucose, their 2-OH group is axial and cannot attack the enzyme-substrate intermediate.

The results of the experiments of the lysozyme catalysed hydrolysis of the trisaccharides are shown in Table I. All the compounds are cleaved between MurNAc and X, although at a very slow rate, with the D-galactose trisaccharide being hydrolysed fastest. This indicates that all the trisaccharides can occupy subsites C D E of lysozyme and span its catalytic site. The finding that the D-galactose-containing trisaccharide is hydrolysed considerably faster than the other trisaccharides tested can be explained by the preferential non productive binding of the latter to subsites A B C of lysozyme (15, 16).

Again, examination of the three dimensional model of the enzyme indicates that GlcNAc β(1→4)-MurNAc β(1→2)-D-Gal can apparently not bind non-productively to subsites A B C because the 4-OH group of its D-galactose moiety makes too close contacts with the enzyme.

Conclusions: (1) Lysozyme can form linkages other than β(1→4) such as β(1→2) or β(1→3). Furthermore, certain β(1→2) linkages, and probably β(1→3) as well, can be cleaved by the enzyme. (2) In order for saccharides to serve as good acceptors in lysozyme catalysed transglycosylation reactions, they have to fit into subsite E of the cleft without unfavorable steric interactions with the enzyme. (3) This fit may necessitate an orientation of the saccharide at subsite E which is different from that proposed for GlcNAc in the three dimensional model of the lysozyme-substrate complex. (4) In addition, to be an acceptor a saccharide must have suitably placed hydroxyl groups to react with the activated lysozyme-saccharide intermediate. (5) Certain L-sugars such as L-glucose and L-fucose which satisfy these steric requirements have been found to be good acceptors.

References:

1. Sharon, N., Proc. Roy. Soc. (London), Ser. B, 167, 402 (1967).
2. Pollock, J.J., Chipman, D.M., and Sharon N., Biochem. Biophys. Res. Commun., 28, 779 (1967).
3. Rupley, J.A., and Gates, V., Proc. Nat. Acad. Sci. U.S.A., 57, 496 (1967).
4. Chipman, D.M., Pollock, J.J., and Sharon, N., J. Biol. Chem., 243, 487 (1968).
5. Blake, C.C.F., Johnson, L.N., Mair, G.A., North, A.C.T., Phillips, D.C., and Sarma, V.R., Proc. Roy. Soc. (London), Ser. B, 167, 378 (1967).
6. Lindberg, B., and Swan, B., Acta Chem. Scand., 14, 1043 (1960).
7. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F., Anal. Chem., 28, 350 (1956).
8. Mirelman, D., and Sharon, N., J. Biol. Chem., 242, 3414 (1967).

9. Leyh-Bouille, M., Ghuysen, J.M., Tipper, D.J., and Strominger, J.L., Biochemistry, 5, 3079 (1966).
10. Schiffman, G., Kabat, E.A., and Leskowitz, S., J. Amer. Chem. Soc., 82, 1122 (1960).
11. Goldstein, I.J., Hay, G.W., Lewis, B.A., and Smith, F., Meth. Carb. Chem., 5, 361 (1965).
12. Pollock, J.J., Chipman, D.M., and Sharon, N., Arch. Biochem. Biophys., 120, 235 (1967).
13. Zehavi U., Pollock, J.J., Teichberg, V.I., and Sharon, N., Nature, 219, 1152 (1968).
14. Raftery, M.A., and Rand-Meir, T., Biochemistry 7, 3281 (1968).
15. Chipman, D.M., Grisaro, V., and Sharon, N., J. Biol. Chem., 242, 4388 (1967).
16. Pollock, J.J., Zehavi, U., Teichberg, V.I., and Sharon, N., Israel J. Chem., 6, 120p (1968).